REMARKS

Claims 101-132 are pending in this application. No amendments to the claims have been made herein.

Claims 101-132 stand rejected under 35 U.S.C. §101 for alleged lack of a "well-established utility." Applicants again respectfully disagree with the rejection, and respectfully request reconsideration of the same in view of the following remarks.

As will be recognized, MPEP §2107.02 II.B states that an invention has a well-established utility if:

(i) a person of ordinary skill in the art would immediately appreciate why the invention is useful based on the characteristics of the invention, and

(ii) the utility is specific, substantial and credible.

As discussed below, the present invention fully meets these criteria.

A. Those of Skill in the Art Would Have Immediately Appreciated Why the Invention is Useful

As Dr. Behan stated in his previously submitted Declaration of June 7, 2004, on the priority filing date of the present application (March 12, 1999), a person of ordinary skill in the art would have immediately appreciated why the invention is useful.[1] For example, Dr. Behan states that:

[1] As a preliminary matter, Applicants note that the Office Action refers to Dr. Behan as "a concerned party" because he is an inventor of the present application. To the extent that the Office Action intended to infer that Dr. Behan's Declaration should be given less consideration because of his status as an inventor, Applicants assert that there is no legal basis to ignore the well reasoned and thoroughly supported evidence provided by a declarant, under penalty of perjury, due to that declarant being either an inventor or a concerned party. Applicants find the Office Action's unjustified attack on Dr. Behan's probity to be particularly troubling as the evidence he has provided is so clearly supported by scientific literature and remains the only evidence on the record.

- **cAMP stimulates thyroid hormone secretion.** Indeed, that cAMP stimulates thyroid hormone secretion was described in scientific papers published in the art.[2]

- **an elevated level of intracellular cAMP in the thyroid leads to an over production of thyroid hormones in Graves' disease**

See Dr. Behan's previously submitted Declaration at paragraph 5, and Goodman and Gilman's The Pharmacological Basis of Therapeutics, McGraw-Hill, Ninth Edition (1996) pages 1383-1409, previously submitted in the Response dated February 12, 2004 as Exhibit A;

- **a compound that could inhibit the production of cAMP in the thyroid could be effective in decreasing the production of thyroid hormones to prevent the exacerbation of, or to treat, Graves' disease; and the present invention could be used to identify such compounds.**

See Dr. Behan's Declaration at paragraph 5, in which he states that biochemists and molecular biologists would have immediately appreciated that the claimed invention directed to the receptor GPR38(V297K) would have been useful because:

(a) the non-endogenous GPR38(V297K) differs from the endogenous GPR38 by a single amino acid, and yields a constitutively active version of the endogenous GPR38;

(b) the constitutively active GPR38(V297K) causes increased production of intracellular cAMP;

(c) GPR38 is expressed in the thyroid;

(d) an activated GPR38 is functionally similar to a GPR38(V297K);

(e) therefore, an activated GPR38 in the thyroid would cause an increase in production of intracellular cAMP therein;

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[2] For example, Murakami et al., Endocrinology (1990) 126: 1692-1698, states that cAMP stimulates thyroid hormone formation (1697, second column) and that cAMP was sufficient to form thyroid hormone (1692, Abstract), attached hereto as Exhibit A. See also Laurberg, Horm. Metab. Res. (1978) 10:152-155, which states that cAMP induces hormone release from the thyroid (153, second column) and that the level of induction was dependent on the concentration of cAMP (153, first column, and 154, Table 1), attached hereto as Exhibit B.

(f) an elevated level of intracellular cAMP in the thyroid leads to an over production of thyroid hormones in Graves' disease;

(g) GPR38(V297K) could have been used in an assay to identify an inverse agonist of GPR38;

(h) such an inverse agonist would have decreased the level of cAMP in the thyroid, and therefore would have been useful to decrease the production of thyroid hormones to prevent the exacerbation of, or to treat, Graves' disease.

Thus, a person of ordinary skill in the art would have immediately appreciated that the claims directed to GPR38(V297K) would have at least one well-established utility, because a GPR38(V297K) could have been employed in screening assays to identify, for example, inverse agonists that could have been used to prevent the exacerbation of, or for the treatment of, Graves' disease.

B. Applicants' Asserted Utility is Specific, Substantial and Credible

1. Applicants' Asserted Utility is Specific

The utility would have been specific because, for example, it was known that GPR38 is specifically expressed in the thyroid, and the inhibition of an activated GPR38 (e.g., by an inverse agonist identified through a screening assay that employs GPR38(V297K)) would have led to a decreased production of cAMP specifically in the thyroid, which in turn could have been used specifically to prevent the exacerbation of, or for the treatment of, Graves' disease. See Dr. Behan's Declaration at paragraph 5.

2. Applicants' Asserted Utility is Substantial

Applicants' asserted utility would have been substantial because the use of GPR38(V297K) in an assay to identify possible inverse agonists thereof, wherein the inverse agonists could have been used to prevent the exacerbation of, or for the treatment of, Graves' disease would have been a "real world" use. In this regard, Applicants note that the Revised Interim Utility Guidelines Training Material (herein after "Training Material") states that "an

assay method for identifying compounds that themselves have a 'substantial utility' define a 'real world' context of use." See page 6 of the Training Material, which was submitted as Exhibit B with Applicant's Response filed on February 12, 2004. In the present case, the compounds that could have been identified in an assay employing GPR38(V297K) would have had substantial utility themselves because these compounds, e.g. inverse agonists of GPR38, could have been administered to prevent the exacerbation of, or for the treatment of, Graves' disease. Thus, the use of GPR38(V297K) in an assay to identify possible inverse agonists thereof also would have been a "real world" use.

3. Applicants' Asserted Utility is Credible

As Dr. Behan states in his Declaration, the asserted utility would have been credible because the use of GPR38(V297K) to screen for inverse agonist compounds, wherein such compounds could have been administered to a patient to prevent the exacerbation of, or for the treatment of Graves' disease, would have been believable to a person of ordinary skill in the art. See paragraph 6 of Dr. Behan's Declaration.

The MPEP dictates that once the applicants have provided a reason for why the claimed invention is useful, the Office personnel may maintain a rejection for alleged lack of utility only if the Office Action establishes that one of ordinary skill would find that the asserted utility is not credible. For example, the MPEP §2107.02 II. B. states that:

> If the applicant subsequently indicates why the invention is useful, Office personnel should review that assertion according to the standards articulated below for review of the credibility of an asserted utility.

The "standard articulated below for review of the credibility of an asserted utility" is found at MPEP §2107.02 III. B, which states:

> Where an applicant has specifically asserted that an invention has a particular utility, that assertion cannot simply be dismissed by Office personnel as being "wrong," even when there may be reason to believe that the assertion is not entirely accurate. Rather, Office personnel must determine if the assertion of utility is credible (i.e., whether the assertion of utility is believable to a person of ordinary skill in the art based on the totality of evidence and reasoning provided).

> An assertion is credible unless (A) the logic underlying the assertion is seriously flawed, or (B) the facts upon which the assertion is based are inconsistent with the logic underlying the assertion. Credibility as used in this context refers to the reliability of the statement based on the logic and facts that are offered by the applicant to support the assertion of utility.

(emphasis in original).

Dr. Behan states in his Declaration that the use of GPR38(V297K) to screen for inverse agonists of GPR38, wherein such inverse agonists could have been administered to prevent the exacerbation of, or for the treatment of, Graves' disease, would have been believable to a person of ordinary skill in the art on March 12, 1999 (the earliest priority date of the present application). See paragraph 6 of Dr. Behan's Declaration. There is no evidence of record that would contradict the logic underlying Dr. Behan's assertion, as set forth in his Declaration, or that would indicate that the facts upon which Dr. Behan's assertion is based are inconsistent with the logic underlying the assertion. Indeed, should the Office question any of the statements of fact in Dr. Behan's Declaration, Applicants would then request that the Office provide evidence to rebut these statements. See *In re Alton*, 76 F.3d 1168 (Fed. Cir. 1996), and The Federal Register, Vol. 66, No. 4, January 5, 2001, pages 1098-1099.

C. The Arguments of the Office Action Have Been Overcome by the Behan Declaration

The Office Action appears to premise its assertion of non-utility on its perception that 1) the specification fails to provide specific details on the mechanism of action of GRP38 (and hence, GPR38(V297K)); and 2) the specification fails state the asserted utility. In this regard, the Office Action asserts that:

- the normal physiological role and endogenous ligand for GPR38 are unknown;
- the G protein that interacts with GPR38 and the mechanism of action of GPR38 (i.e., the identity of the specific G protein to which GPR38 couples, etc.) are unknown; and
- neither the asserted utility nor information relating to the mechanism of action of the receptor were disclosed in the specification.

Specifically, the Office Action states that the specification does not disclose the relationship between GPR38, cAMP levels and Graves' disease, and, more specifically, the biological significance of GPR38, the utility of GPR38 or GPR38(V297K) to prevent the exacerbation of, or for the treatment of, Graves disease, the agonist that interacts with GPR38 and decreases cAMP levels, whether GPR38 or GPR38(V297K) is overexpressed or underexpressed in Graves' disease; or that changing cAMP levels by modulating GPR38 would have any effect on Graves' disease, or that GPR38 increases cAMP levels upon binding ligand.[3]

With due respect, the Office Action has misapplied the criteria for determining utility of the present claims. Applicants are simply not required to provide the information the Office Action seeks.

1. Applicants Are Not Required To State A Well Established Utility In Their Specification.

To show that the invention has a well established utility, all that is necessary is that Applicants provide evidence that those of skill in the art would have recognized that utility, i.e., that ***a person of ordinary skill in the art would immediately appreciate why the invention is useful based on the characteristics of the invention, and that the utility is specific, substantial and credible***. The patent laws require no more. In the present case, as discussed above, Dr. Behan's Declaration establishes that a person of ordinary skill in the art would have immediately appreciated that the claims directed to GPR38(V297K) would have at least one well-established utility, because a GPR38(V297K) could have been employed in screening assays to identify, for

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[3] Applicants note that the Office Action additionally asserts:

> ... any compound could be considered a regulator or modulator of tissue in that any compound, if administered in the proper amount, will stimulate or inhibit tissue. For example, salt, ethanol, and water area all compounds which kill cells if administered in a great enough amount, and which would stimulate cells from which these compounds had been withheld, therefore, they could be considered regulators or modulators of tissue.

Office Action at page 9. However, as discussed above and in Dr. Behan's Declaration, GPR38(V297K) could have been employed in a screening assay to identify compounds, for example inverse agonists, that can inhibit the activity of a ***specific*** receptor (GPR38) that is localized in a ***specific*** tissue (the thyroid). Thus, the comparison proposed by the Office Action is completely inappropriate.

example, inverse agonists that could have been used to prevent the exacerbation of, or for the treatment of, Graves' disease. Also as discussed above, Dr. Behan's Declaration and the Training Material establish that the utility is specific, substantial and credible. See section B of this paper, above. Thus, those of skill in the art ***would have immediately appreciated*** that the invention was useful for the presently asserted utility ***based on the characteristics of the invention***. It is therefore ***irrelevant*** whether or not the specification specifically states the asserted utility. Indeed, it is ***implicit in the very definition of the "well established utility" that those of skill in the art would know the utility from the character of the invention without recourse to the specification***. Accordingly, Applicants have met the requirements for showing the presently asserted well established utility.[4]

2. The Information Sought By The Office Action Is Not Required To Show The Invention Possessed A Well Established Utility

It can be seen from the discussion above that, contrary to the assertion of the Office Action, none of the information sought by the Office Action is required to show that the invention possessed a well established utility. Simply put, Dr. Behan's Declaration establishes that those of skill in the art would have appreciated the asserted utility, and that the utility is specific, substantial and credible. Thus, it is not required that the specification disclose the information sought by the Office Action. Indeed, whether or not the specification discloses the relationship between GPR38, cAMP levels and Graves' disease, the biological significance of GPR38, the utility of GPR38 or GPR38(V297K) for treating or preventing Graves' disease, the agonist that interacts with GPR38 and decreases cAMP levels, whether GPR38 or GPR38(V297K) is overexpressed or underexpressed in Graves' disease, that changing cAMP levels by modulating GPR38 would have any effect on Graves' disease, or that GPR38 increases cAMP levels upon binding ligand is ***irrelevant*** because Dr. Behan's Declaration makes it clear that those of skill in the art would have appreciated why the invention is useful based on the

[4] Indeed, Applicants note that either an explicitly stated utility, ***or*** an asserted specific and substantial utility is sufficient to satisfy 35 USC § 101. Indeed, the title of MPEP § 2107.02 II.B itself reads: "No statement of utility for the claimed invention in the specification does not per se negate utility". Thus, Applicants' assertion that the claimed invention has a well-established utility is sufficient to meet the requirements of 35 USC § 101.

characteristics of the invention. Inasmuch as the Office Action has not provided any evidence that those of ordinary skill would doubt the credibility of the facts asserted by Dr. Behan in his Declaration, the present invention must be deemed to have the asserted well-established utility.[5]

In view of the preceding remarks, Applicants respectfully request the rejections under 35 U.S.C. § 101 be withdrawn.

Claims 101-132 also stand rejected under 35 U.S.C. § 112, first paragraph, for alleged lack of enablement, on the basis that the claims allegedly lack utility. In light of the arguments above, Applicants respectfully submit that those skilled in the art would recognize both the utility of the invention and how to use it. Moreover, methods of screening for modulators of GPCRs were well known in the art at the priority filing date of the present application, and such methods were described in the earliest priority document. See, for example, Section C (page 18, line15, to page 20, line 19) and Example 4 (page 24, line 19, to page 25, line 17) of Provisional Application 60/123,945, filed on March 12, 1999. Applicants therefore respectfully request withdrawal of the rejection under 35 U.S.C. § 112, first paragraph.

In conclusion, Applicants respectfully assert that the claimed inventions directed to GPR38(V297K) have a well-established utility, and that the Office Action has not provided any reason for one of ordinary skill in the art to doubt the credibility of such utility. Accordingly, Applicants respectfully request a withdrawal of the rejection under 35 U.S.C. § 101 and § 112, first paragraph. Further, Applicants assert that the claims are in condition for allowance, and respectfully request notification to that effect.

[5] To the extent that the Office Action intends to question the veracity of Applicants' assertion regarding the effect of changing cAMP levels on Graves' disease, Applicants respectfully assert that the burden is on the Office to provide reasons why those of skill in the art would question Dr. Behan's statement.

Should the Office have any questions, Applicants invite the Office to contact the undersigned at (215) 665-2158 to discuss any issues unresolved by this Amendment. A Notice of Allowance is earnestly solicited.

Respectfully submitted,

Date: September 13, 2004

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0013-7227/90/1263-1692$02.00/0
Endocrinology

Exhibit A

Vol. 126, No. 3
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Physiological *de Novo* Thyroid Hormone Formation in Primary Culture of Porcine Thyroid Follicles: Adenosine 3′,5′-Monophosphate Alone Is Sufficient for Thyroid Hormone Formation*

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ABSTRACT. We describe a method of culturing intact porcine thyroid follicles for physiological *de novo* thyroid hormone formation; the roles of cAMP and protein kinase-C in thyroid hormone formation were also studied. Thyroid follicles were obtained by digesting minced porcine thyroid tissue with 0.04% collagenase and cultured in Coon's Modified Ham's F-12 medium supplemented with 0.6% calf serum, 0.5 mU/ml TSH, other standard hormones, and 3 antibiotics (6H medium). On the fourth day of culture, 6000–8000 follicles/well were plated in 12-well culture dishes. On the sixth day, thyroid hormone formation was carried out by incubating thyroid follicles with 0.5 μM KI in the presence of 6H medium for 2 days in a 5% CO_2–95% air incubator at 37 C. To examine the effects of cAMP and protein kinase-C on *de novo* thyroid hormone formation, follicles were incubated with KI in the presence of 1–2.5 mM $(Bu)_2$cAMP, 10 μM forskolin, 2 μM prostagladin E_2 (PGE_2), or 0.5–1 μM 12-*O*-tetradecanoylphorbol-13-acetate in TSH-free medium for 2 days. The amount of newly formed thyroid hormone was measured by RIA of T_3 content in the Pronase digest of thyroid follicular cells. Thyroid follicles cultured in 6H medium had normal polarity of the membrane, determined by electron microscope, and thyroid cAMP was responsive to the alteration of TSH. In this culture system cAMP alone was sufficient to form thyroid hormone. 12-*O*-Tetradecanoylphorbol-13-acetate, a protein kinase-C stimulator, disrupted thyroid follicles and inhibited cAMP-mediated thyroid hormone formation. The integrity of follicular structure was also required for thyroid hormone formation in this culture system.

This study introduces perhaps the most physiological culture system for *de novo* thyroid hormone formation. Our data provide direct evidence that thyroid hormone formation is linked to cAMP and that the protein kinase-C system acts as an inhibitor of thyroid hormone formation. (*Endocrinology* 126: 1692–1698, 1990)

THE MAIN function of thyroid cells is to form thyroid hormone. However, thyroid cells in culture generally do not form thyroid hormone. This is because most thyroid cells in culture do not form an intact follicular dome structure, a functional unit for hormone formation, and lose their original cell function (1–4). In primary culture, only sheep thyroid cells (5) and human thyroid follicles (6) have shown *de novo* thyroid hormone formation. In the established thyroid cell lines, GEJ human thyroid cells have produced thyroid hormone (7). However, thyroid hormone formation by sheep thyroid cells and GEJ cells probably does not represent a physiological event because of the lack of intact follicular structure and an undetermined polarity of the membrane. Suspension culture of human thyroid follicles in the presence of dimethylsulfoxide demonstrated *de novo* thyroid hormone formation by TLC (6). Unfortunately, human thyroid gland tissue is not easily available, and the method of demonstrating the quantity of newly formed thyroid hormone may not be suitable for multiple sampling. We sought a simple and physiological culture system in which *de novo* thyroid hormone formation can be studied. We chose easily available porcine thyroid tissue as a source of thyroid follicles and demonstrated physiological *de novo* thyroid hormone formation. Using this culture system, the basic conditions of thyroid hormone formation, including the roles of the cAMP system and the protein kinase-C system, were examined by correlating them with the morphological changes in thyroid follicles.

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Materials and Methods

Reagents

Enzymes, hormones, and other reagents were obtained from Sigma Chemical Co. (St. Louis, MO) unless otherwise specified.

Isolation of thyroid follicles

Porcine thyroid glands were obtained from a local slaughterhouse and brought to the laboratory immediately in ice-cold Ca^{2+}- and Mg^{2+}-free Hanks' Balanced Salt Solution. The thyroid tissues were trimmed free of capsule, fat, and connective tissue and finely chopped into small pieces. The chopped tissue was washed 4 times with Hanks' Balanced Salt Solution that contained penicillin (10,000 U/ml), streptomycin (10 mg/ml), and amphotericin-B (25 μg/ml). Digestion of thyroid tissue was carried out in 80 ml serum-free Coon's Modified Ham's F-12 medium (Irvine Scientific, Santa Ana, CA) containing 0.04% collagenase (type V; Sigma Chemical Co.) and 3 antibiotics, described above. The mixture of collagenase and thyroid tissue was kept at 37 C for 1 h, and thyroid tissue was triturated with a 10-ml pipet every 10 min for another 1 h to release thyroid follicles. The released follicles were obtained by filtering the collagenase mixture through a nylon mesh (200-μm pore size) and centrifuging the filtered materials at 50 × *g* for 2 min. The pellet containing follicles was suspended in 6H medium that consisted of Coon's Modified Ham's F-12 medium, 0.5% calf serum, 0.5 mU/ml bovine TSH, 10 μg/ml insulin, 5 μg/ml transferrin, 10 ng/ml somatostatin, 10 ng/ml glycine-L-histidyl-L-lysine, 10 μM hydrocortisone, and 3 antibiotics (8). The thyroid follicles were washed 4 times with 6H medium, plated in a 75-cm^2 flask, and cultured in 6H medium at 37 C in a humidified 5% CO_2–95% air incubator. Culture medium was changed every day for the first 4 days to remove the released thyroid hormone in the medium. On the fourth day, thyroid follicles were removed by gentle pipetting and plated into 12-well culture dishes at a density of 8000 follicles/well. It is essential to disperse the follicles, since aggregated follicles are unsuitable for *de novo* thyroid hormone formation. Thyroid follicles were kept for 2 days in 6H medium before initiation of *de novo* thyroid hormone formation.

Thyroid hormone formation in cultured follicles

On the sixth day of culture, follicles were washed with 6H medium, and a final volume of 1 ml 6H medium was added. Thyroid hormone formation was started by adding KI at a final concentration of 0.5 μM. Incubation was performed in triplicate wells for 2 days at 37 C in a humidified 5% CO_2–95% air incubator. At the end of the incubation, culture medium was removed, and 1 ml 0.05 M sodium phosphate buffer, pH 7.0, was added. Cells supended in phosphate buffer were sonicated and centrifuged at 13,000 × *g* for 30 min. The supernatant (300 μl) was digested with 50 μl Pronase (12 mg/ml) for 18 h at 37 C under nitrogen (9). The thyroid hormone content in the Pronase digest was measured by T_3 RIA kit (Diagnostic Products Corp., Los Angeles, CA), and the results were expressed as picograms of T_3 per μg DNA.

To examine the effects of TSH, cAMP, and phorbol ester on *de novo* thyroid hormone formation, thyroid follicles were washed with TSH-free medium (5H medium) twice on the sixth day of culture. Then, 1 ml 5H medium containing 0–2 mU/ml TSH, 0.01–1 mM $(Bu)_2$cAMP, 25 μM forskolin, 2 μM prostaglandin E_2 (PGE_2), and 0.5–1 μM 12-*O*-tetradecanoylphorbol-13-acetate (TPA) was added immediately after washing. Thyroid hormone formation was started by adding 0.5 μM KI to the medium, and incubation was carried out for 2 days as described above. PGE_2 was also used as a generator of cAMP (10). To examine the effects of different concentrations of KI on thyroid hormone formation, 0–10 μM KI was added to the 6H medium for *de novo* thyroid hormone formation.

Measurement of cAMP

To examine the biological response of thyroid follicles to the alteration of TSH, the intracellular cAMP level was measured in the presence and absence of TSH. Thyroid follicles (8000 follicles/well) cultured in 6H medium for 6 days were washed twice with 5H medium, and 1 ml 5H medium was added. Samples for measurement of intracelullar cAMP levels were obtained 0, 4, 12, and 24 h after withdrawal of TSH. To examine the generation of cAMP in response to TSH, thyroid follicles on the sixth day of culture were exposed to 5H medium for 24 h, and then 0.5 mU/ml TSH was added. Samples for cAMP assay were obtained before addition of TSH and 4 h after addition of TSH. To measure cAMP in follicular cells, the medium was removed, and 1 ml 95% ethanol-120 mM HCl was added; this mixture was kept overnight at −20 C. The samples were dried under N_2 gas, and 200–300 μl 50 mM sodium acetate buffer, pH 6.2, were added. The intracellular cAMP level was measured from an aliquot using a RIA kit (Incstar Co., Stillwater, MN).

Electron microscopy

Electron microscopy was used to assess the polarity of the membrane of thyroid follicular cells. Fixation was carried out with 2% glutaraldehyde in 0.05 M PBS, pH 7.4, for 1 h at room temperature, and the follicles were postfixed using 1% osmium tetraoxide in PBS for 1 h. The fixed follicles were dehydrated through graded steps of ethanol treatment and embeded in Polybed resin (Polysciences, Warrington, PA). Ultrathin sections were cut with a LKB ultramicrotome (LKB, Bromma, Sweden) and mounted on copper grids. After staining with 4% uranyl acetate and Reynold's lead citrate, the tissue was examined using Zeiss 109 transmission electron microscope (New York, NY).

DNA assay

The DNA content in cell sonicate was measured by a mithramycin spectrofluorimetric method (11).

Statistical analyses

The significance of the differences of the mean values was analyzed by unpaired Student's *t* test.

Results

Morphology of thyroid follicles

Figure 1A shows thyroid follicles in the presence of TSH (0.5 mU/ml). The thyroid follicles had a dome shape that contained a closed follicular lumen, since 0.4% trypan blue dye did not penetrate into the follicular

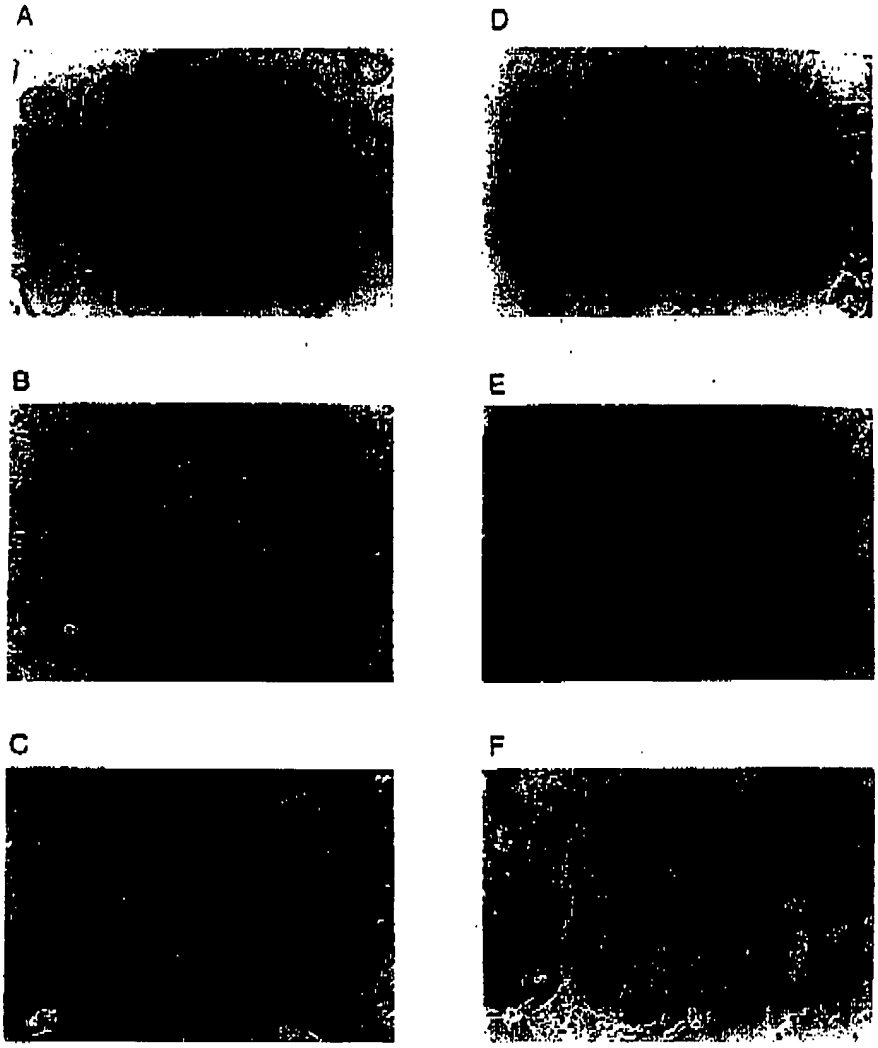

FIG. 1. A, Thyroid follicles cultured in 6H medium. B, Reorganized thyroid follicles when thyroid cells were cultured in a monolayer for 3 weeks in 6H medium. Cells surrounding a follicle were thyroid cells. C, Thyroid follicles exposed to 5H medium (TSH-free medium) for 24 h. D and E, Thyroid follicles cultured in the presence of 1 mM $(Bu)_2$cAMP and 1 μM PGE_2, respectively, in 5H medium. F, Thyroid follicles exposed to 0.5 μM TPA for 24 h in 6H medium.

lumen. Electron microscopic examination showed the presence of microvilli in the apical border of the plasma membrane (Fig. 2). We viewed at least 10 follicles, and none of them had reversed polarity of the membrane. When individual thyroid cells were cultured in a monolayer for 3–4 weeks, reorganized follicles began to appear (Fig. 1B). In contrast to the primary follicles, which were present from the beginning of culture, the number of reorganized follicles is small. Figure 1C depicts thyroid follicles cultured in 5H medium for 24 h. Characteristically, follicular lumen disappeared, and follicles were flattened without TSH. When 1 mM $(Bu)_2$cAMP (Fig. 1D) or a cAMP-generating system such as 1 μM PGE_2 (Fig. 1E) was added to the TSH-free medium (5H medium), follicular structure was well maintained. The presence of 0.5 μM TPA in 6H medium for 24 h caused disruption of the follicular structure and a loss of follicular lumen; individual cells began to proliferate without forming follicles (Fig. 1F).

Thyroid hormone formation

We chose the sixth day of culture to initiate *de novo* thyroid hormone formation, since previously formed thyroid hormone in the follicular cells was depleted by the sixth day, and *de novo* thyroid hormone formation was best demonstrated when KI was added at this point. Figure 3 shows the time course of thyroid hormone formation after adding KI. The maximum hormone formation in the follicular cells was obtained on the second day of incubation. Thyroid hormone content in the medium was at its maximum on the third day, indicating that there was a lag period to secrete thyroid hormone from the cells to the medium. The presence of 10 μM

FIG. 2. Electron microscope view of thyroid follicles. L, Follicular lumen. Microvilli are seen in the membrane of follicular lumen site.

methimazole or 20 μM propylthiouracil inhibited *de novo* thyroid hormone formation completely in this system (data not shown).

Effect of TSH on de novo thyroid hormone

Figure 4 shows the amount of thyroid hormone formed in response to different TSH concentrations. There was a progressive increase in T_3 formation in the follicular cells when the TSH concentration was raised. A significant amount of T_3 formation was seen even with 10 μU/ml TSH. The maximum T_3 formation in cells occurred at TSH concentration greater than 0.5 mU/ml. The T_3 content in the medium did not differ significantly in the presence or absence of TSH. Thus, *de novo* thyroid hormone formation can be best demonstrated in the cells and not in the medium when hormone formation is determined 2 days after the addition of KI.

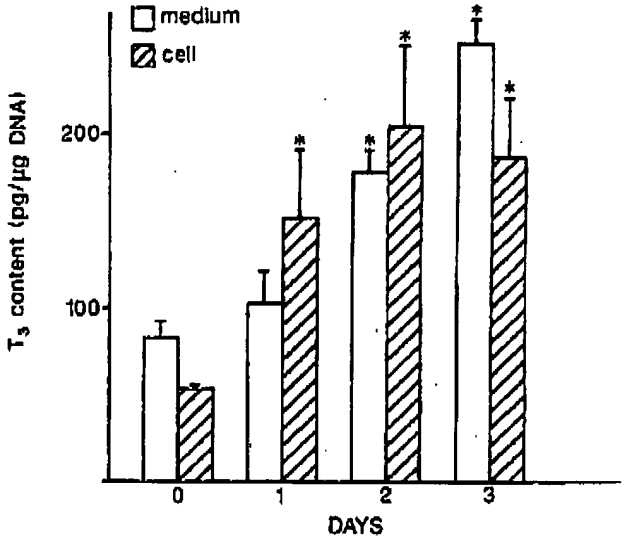

FIG. 3. Time course of thyroid hormone formation. The T_3 content on day 0 indicates the amount of T_3 present in thyroid follicular cells before initiating thyroid hormone formation. *, $P < 0.01$ compared to the basal T_3 content on day 0. The results are the mean ± SD of triplicate wells.

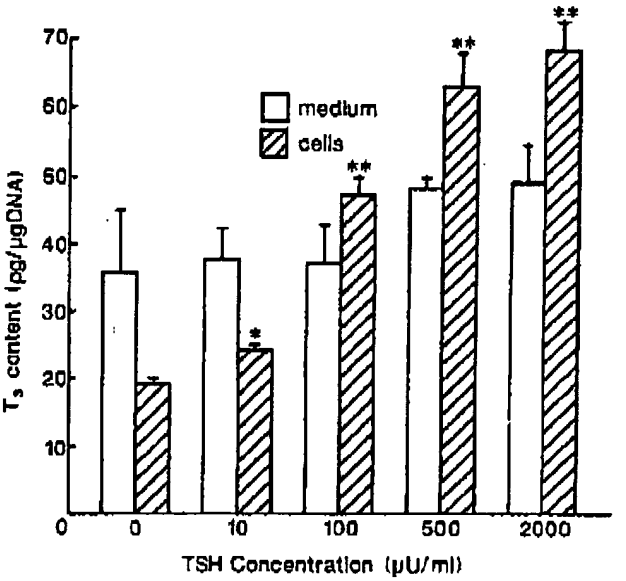

FIG. 4. Thyroid hormone formation in response to different concentrations of TSH. The results are the mean ± SD of triplicate wells. Thyroid follicles were incubated with 0.5 μM KI for 2 days in the presence of 0–2000 μU/ml TSH. *, $P < 0.05$; **, $P < 0.01$ (compared to the T_3 content of follicles incubated without TSH).

Effect of KI on de novo thyroid hormone formation

Figure 5 shows the amount of T_3 formation in response to 0.1–10 μM KI. The optimal concentrations of KI for thyroid hormone formation were between 0.1–0.5 μM.

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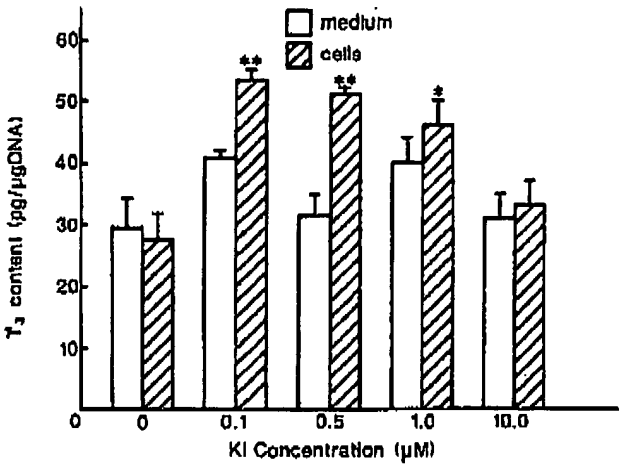

FIG. 5. Thyroid hormone formation in response to different KI concentrations. Incubation for thyroid hormone formation was carried out for 2 days in the presence of 0.5 mU/ml TSH. The results are the mean ± SD of triplicate wells. *, $P < 0.02$; **, $P < 0.01$ (compared to the T_3 content of follicles incubated without KI).

There was a significant decrease in T_3 formation when the KI concentration was greater than 10 μM.

Effect of calf serum

When follicles were cultured with less than 0.1% calf serum, thyroid follicles did not attach to the plate. The amount of thyroid hormone formed did not differ significantly when the calf serum concentration was raised from 0.5% to 5% (data not shown). To negate the adverse effect of high serum concentration on hormone formation, 0.5% calf serum was used throughout the experiment.

Roles of cAMP and protein kinase-C in thyroid hormone formation

Since cAMP (12–14) and protein kinase-C (15–21) affect thyroid cell function, differentiation, and growth, we examined the effects of $(Bu)_2$cAMP, forskolin, and TPA on thyroid hormone formation. As shown in Fig. 6, 1 mM $(Bu)_2$cAMP and 25 μM forskolin caused 3.2- and 3.5-fold increases in T_3 formation, respectively, indicating that cAMP is involved in *de novo* thyroid hormone formation. The presence of TPA, a stimulator of protein kinase-C, in TSH-free medium did not stimulate thyroid hormone formation. In fact, TPA inhibited cAMP-mediated thyroid hormone formation. Next, we examined whether the cAMP system alone is equally effective as TSH for the formation of thyroid hormone. Indeed, 2.5 mM $(Bu)_2$cAMP, 25 μM forskolin, and 2 μM PGE_2 produced the same amount of T_3 as did 0.5 mU/ml bovine TSH (Fig. 7).

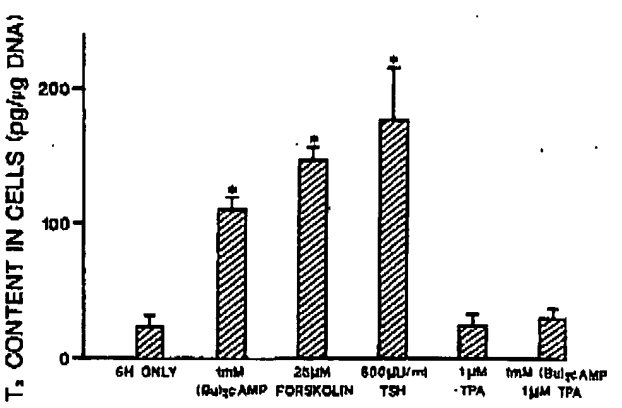

FIG. 6. Thyroid hormone formation in the presence of cAMP, cAMP-generating system, and TPA. Incubation for thyroid hormone formation was carried out in the presence of 0.5 μM KI. The results are the mean ± SD of triplicate wells. *, $P < 0.01$ compared to the T_3 content of follicles incubated in 5H medium.

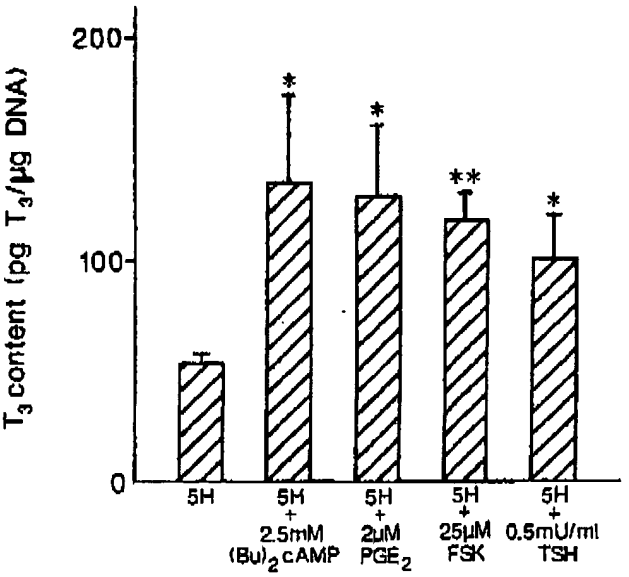

FIG. 7. Efficacy of the cAMP system for thyroid hormone formation. The results are the mean ± SD of triplicate wells. *, $P < 0.02$; **, $P < 0.01$ (compared to the T_3 content of follicles incubated in 5H medium). FSK, Forskolin.

Responsiveness of thyroid cAMP to the alteration of TSH

We also examined whether cAMP in thyroid follicles can respond to the alteration of TSH. The basal cAMP level in the presence of 0.5 mU/ml TSH was 11.5 ± 3.4 pmol/well (mean ± SD of triplicate wells). Four hours after withdrawal of TSH, cAMP levels decreased to 3.2 ± 0.7 pmol/well and remained at the same low level for 24 h. Four hours after the addition of TSH to the follicles cultured in TSH-free medium for 24 h, the thyroid cAMP level rose from 2.8 ± 0.8 to 10.0 ± 3.0 pmol/well.

Discussion

Our culture system showed *de novo* thyroid hormone formation in porcine thyroid follicles. The amount of newly formed T_3 in follicular cells was used as an index of *de novo* thyroid formation. The amount of newly formed T_4 during incubation reflected an increment of 2–3 μg T_4/dl by T_4 RIA. In contrast, the T_3 RIA showed changes in T_3 content from 50 to 200 ng/dl. Therefore, the increment of thyroid hormone formation was best demonstrated by the T_3 assay rather than the T_4 assay. The method described here is probably the most suitable for the study of *de novo* thyroid hormone formation in the cell culture system. First, porcine thyroid tissue is easily available. Second, thyroid hormone formation is a physiological event because of intact follicle and normal membrane polarity. Third, the amount of thyroid hormone formed is demonstrated by RIA rather than using time-consuming chromatographic analysis. Fourth, this simple culture system is suitable for experiments requiring multiple samples.

Our study clearly showed the importance of intact follicular structure for *de novo* thyroid hormone formation. Disruption of follicular structure caused a significant decrease in thyroid hormone formation. The lack of TSH and the presence of TPA are examples of follicular disruption. Therefore, inspection of thyroid follicles through a microscope provides us some idea of whether hormone formation occurs. The follicles used in this experiment had normal polarity of the membrane, as judged by an electron microscope. It has been reported that porcine thyroid follicles cultured in the presence of the cAMP system from the beginning of culture can maintain their original polarity of the membrane (3). We have not examined the function of reorganized follicles for the formation of thyroid hormone. However, reorganized follicles may not be suitable for studying *de novo* thyroid hormone formation, because the number of reorganized follicles is generally small, and the membrane may have a reversed polarity, such as the inside-out picture (4).

Using this culture system, we studied the controlling factors of thyroid hormone formation in cultured thyroid follicles. Our results showed that *de novo* thyroid hormone formation in this culture system required TSH and the optimal concentration of KI. Since TSH action can be mediated through cAMP and non-cAMP pathways (18, 21), we examined which pathway is the most important for thyroid hormone formation. TSH was substituted with $(Bu)_2$cAMP or cAMP-generating systems such as forskolin and PGE_2; then, the efficacy of the cAMP system on *de novo* thyroid hormone formation was examined by comparing it with that of TSH. Our results showed that the cAMP system alone was as efficient as TSH for thyroid hormone formation. The most well known function of cAMP in the thyroid gland is to stimulate thyroid hormone secretion (22, 23). Surprisingly, the direct linkage between cAMP and thyroid hormone formation has not been demonstrated clearly. Our study provides the first direct evidence that thyroid hormone formation is indeed linked to cAMP. There are several fragmented data suggesting that cAMP participates in thyroid hormone formation. For instance, cAMP has been shown to stimulate thyroid peroxidase activity in the primary culture of dog thyroid cells (1), iodide uptake in FRTL-5 cells (24), and thyroglobulin mRNA formation (25). TSH is also needed to maintain intact follicular structure (12). Therefore, it is not surprising to see the stimulatory action of cAMP on thyroid hormone formation. A study reported by Karsenty *et al.* (7), however, is noteworthy. Their GEJ human thyroid cells produced thyroid hormone independent of cAMP. Factors other than cAMP have been speculated to initiate thyroid hormone formation in their specific cells.

The thyroid gland is also under the control of the protein kinase-C system (15–21). TPA, a protein kinase-C stimulator, has been shown to stimulate iodide incorporation into proteins, glucose oxidation, and ^{32}P incorporation into phospholipids (18). However, the effect of TPA on the overall thyroid hormone formation has not been studied. Our data indicate that the protein kinase-C system appears to act as an inhibitor of thyroid hormone formation. The mechanism of TPA-mediated inhibition of thyroid hormone formation can be explained by the following two findings: disruption of the thyroid follicular structure and inhibition of cAMP-dependent thyroid hormone formation. TPA has been shown to inhibit both TSH-dependent cAMP generation and cAMP-mediated action at a post-cAMP locus (21). Our finding of TPA-induced inhibition of thyroid hormone is in agreement with such reports.

In summary, *de novo* thyroid hormone formation has been difficult to demonstrate in the culture system, but our culture system overcame such difficulties and showed *de novo* thyroid hormone formation in easily available porcine thyroid follicles. This culture system should be useful for measurement of the biological activity of TSH, cAMP, and antithyroid agents by the amount of newly formed thyroid hormone.

References

1. Magnusson RP, Rapoport B 1985 Modulation of differentiated function in cultured thyroid cells: thyrotropin control of thyroid peroxidase activity. Endocrinology 116:1493
2. Denef JF, Björkman U, Ekholm R 1980 Structural and functional characteristics of isolated thyroid follicles. J Ultrastruct Res 71:185
3. Mauchamp J, Margotat A, Chambard M, Charrier B, Remy L, Michel-Bechet M 1979 Polarity of three demontional structure derived from isolated hog thyroid cells in primary culture. Cell Tissue Res 204:417

4. Karlsson FA, Westermark K, Westermark B 1982 Functional properties of porcine thyroid follicles in suspension. Mol Cell Endocrinol 28:995.
5. Becks GP, Eggo MC, Burrow GN 1987 Regulation of differentiated thyroid function by iodide: preferential inhibitory effect of excess iodide on thyroid hormone secretion in sheep thyroid cell cultures. Endocrinology 120:2569
6. Sato K, Han DC, Ozawa M, Fujii Y, Tsushima T, Shizume K 1988 Dimethylsulfoxide maintains human thyroid cells in suspension culture, facilitating synthesis and release of thyroid hormone. Biochem Biophys Res Commun 155:100
7. Karsenty G, Aliguier C, Jelsema C, Weintraub BD 1988 Thyrotropin induces growth and iodothyronine production in a human thyroid cell line without affecting adenosine 3′,5′-monophosphate production. Endocrinology 123:1977
8. Ambesi-Impiombato FS, Parks LAM, Coon HG 1980 Culture of hormone-dependent functional epithelial cells from rat thyroids. Proc Natl Acad Sci USA 77:3455
9. Inoue K, Taurog A 1967 Digestion of ^{131}I-labeled thyroid tissue with maximum recovery of ^{131}I-iodothyronines. Endocrinology 81:319
10. Spaulding SW, Burrow GN 1978 Effect of PGE_1 and TSH on cAMP-dependent protein kinase activity in the thyroid. Endocrinology 96:1018
11. Hill BT, Whatley S 1975 A simple rapid microassay for DNA. FEBS Lett 56:20
12. Bourke JR, Matsinaho T, Huxham GJ, Manley SW 1987 Cyclic AMP-stimulated fluid transport in the thyroid: influence of thyroid stimulators, amiloride and acetazolamide on the dynamics of domes in monolayer cultures of porcine thyroid cells. J Endocrinol 115:19
13. Roger PP, Dumont JE 1984 Factors controlling proliferation and differentiation of canine thyroid cells cultured in reduced serum conditions: effects of thyrotropin, cyclic AMP, and growth factors. Mol Cell Endocrinol 36:79
14. Roger PP, Hotimsky A, Moreau C, Dumont JE 1982 Stimulation by thyrotropin, cholera toxin, and dibutyryl cyclic AMP of multiplication of differentiated thyroid cell function *in vitro*. Mol Cell Endocrinol 26:165
15. Lippes HA, Spaulding WS 1986 Peroxide formation and glucose oxidation in calf thyroid slices: regulation by protein kinase-C and cytosolic free calcium. Endocrinology 118:1308
16. Philip NJ, Grollman FF 1986 Thyrotropin and norepinephrine stimulate the metabolism of phosphoinositides in FRTL-5 cells. FEBS Lett 202:193
17. Levasseur S, Morgan L, Friedman Y, Burke G 1985 Forskolin and 12-*O*-tetradecanoyl phorbol-13-acetate mimic thyrotropin-stimulated protein iodination in mouse thyroid. Biochem Biophys Res Commun 128:858
18. Yoshimura Y, Dekker A, Ferdows M, Rani CSS, Field JB 1986 Effects of phorbol esters on metabolic variables in the thyroid. Endocrinology 119:2018
19. Bachrach LK, Eggo MC, Mak WW, Burrow GN 1985 Phorbol esters stimulate growth and inhibit differentiation in cultured thyroid cells. Endocrinology 116:1603
20. Ginsberg J, Murray PG 1986 Protein kinase activators modulate differentiated thyroid function *in vitro*. FEBS Lett 206:309
21. Lombardi A, Veneziani BM, Tramontano D, Ingbar SH 1988 Independent and interactive effects of tetradecanoyl phorbol acetate on growth and differentiated functions of FRTL-5 cells. Endocrinology 123:1544
22. Melander A, Ranklev E, Sundler F, Westgren U 1975 Beta$_2$-adrenergic stimulation of thyroid hormone secretion. Endocrinology 97:332
23. Ahren B, Aluments J, Ericsson M, Fahrenkrug J, Hakanson R, Hedner P, Loren I, Melander A, Rerup C, Sundler F 1980 VIP occurs in intrathyroidal nerves and stimulates thyroid hormone secretion. Nature 287:343
24. Weiss SJ, Philip NJ, Ambesi-Impiombato FS, Grollman EF 1984 Thyrotropin-stimulated transport of iodide mediated by adenosine 3′,5′-monophosphate and dependent on protein synthesis. Endocrinology 114:1099
25. Kung AWC, Collins K, Banga JP, McGregor AM 1988 Effect of Graves' IgG on gene transcription in human thyroid cell cultures. FEBS Lett 232:12

Exhibit B

Horm. Metab. Res. 10 (1978) 152–155

Dynamics of Hormone Release from the Perfused Canine Thyroid during Cyclic AMP Infusion

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Summary

In order to try to characterize the sequence of processes leading to hormone secretion from the stimulated thyroid, the effect of cyclic 3'5' adenosine monophosphate (cAMP) and related compounds was examined in 15 two-sided perfusions of canine thyroids isolated in situ. T_4 and T_3 concentrations in the effluent were measured radioimmunologically.

cAMP 5 mM and TSH 100 μU per ml induced the same pattern of hormone release from the thyroid. After a latency period of 15-25 minutes a steep increase occurred in both T_4 and T_3 release. During the initial part of the stimulation the rise in T_4 release was somewhat slower than that of T_3 release.

The prolonged latency period before response earlier recorded in the same preparation during infusions of low concentrations of TSH was not observed during infusions of decreasing concentrations of cAMP (1, 0.8, 0.5 and 0.2 mM) or theophylline (5 and 1 mM). Either there was no response or the latency period was of the same length as that observed after a strong stimulus. These findings suggest that the latency period can be divided in two parts: 1) a variable, dose dependent latency period confined to the early part of the process sequence leading to secretion – i.e. before cAMP exerts its effect, and 2) an obligatory latency period related to the processes taking place after the formation of pseudopods. The duration of these late processes seems to be independent of the degree of stimulation.

Key-Words: *Cyclic AMP – Thyroid Perfusion – Thyroid Secretion – Thyroid Latency*

Introduction

It is generally accepted that cyclic 3'5' adenosine monophosphate (cAMP) plays a central role in the mediation of many of the effects of TSH on the thyroid. TSH activates the thyroid adenyl cyclase and increases the concentration of cAMP in the thyroid. In addition cAMP or dibutyryl cAMP has the ability to mimic most of the effects of TSH, and phosphodiesterase inhibitors which inhibits the degradation of endogenous cAMP stimulate the thyroid or enhance the effect of TSH. For reviews see *Dumont* 1971, *Field* 1975.

Recently we have described the dynamics of T_4 and T_3 release from a perfused thyroid during infusions of various concentrations of TSH (*Laurberg* 1977). It was found that the duration of the latency period – i.e. the time elapsing from addition of TSH to the perfusion medium until augmentation of hormone release occurs – depended on the concentration of TSH in the perfusion medium. Following 100 μU TSH per ml the latency period was 15-25 min while 2 μU TSH per ml induced a more gradual and delayed response, the latency period being 40-70 min. In order to characterize further the sequence of processes leading to hormone secretion from the stimulated thyroid we have examined the T_4 and T_3 release during infusion of cAMP and compared the effect of cAMP to that of TSH.

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Material and Methods

Fifteen mongrel dogs weighing 16-27 kg were used for the study. The two separate thyroids were isolated and perfused in situ as described previously (*Laurberg* 1976, *Laurberg* 1977). The perfusion pressure was constant at 30-40 mmHg throughout the experiments and was not affected by the infusion of nucleotides. In different experiments the high dose of cAMP or TSH was infused alternatively in the left and right thyroid. All the perfusion lasted for 200 min. The first sample was obtained after 30 min perfusion. The compounds to be examined were diluted in perfusion medium shortly before use and infusion started after 60 min perfusion with control medium. The compounds infused in different experiments appear from Table I.

T_3 and T_4 in effluent were determined by single antibody wick-chromatographic radioimmunoassays (*Weeke and Ørskov* 1975, *Weeke and Ørskov* 1978). Theophylline induced a decrease in the binding of T_3 to the T_3 antibody. Therefore only T_4 was measured in experiments including theophylline. The other compounds tested did not interfere in the assays.

Five minutes samples were continously collected throughout the experiments. Generally, T_3 and T_4 were measured in every sample for a 30 min period following the start of infusion of a compound to be examined. Apart from this, hormones were measured in every second sample.

In all the experiments where signs of stimulation were observed, there was a steep progressive increase in the concentration of hormones in the following series of samples. The latency period was defined as the time elapsed between addition of stimulant and collection of the last sample before increasing values, had occurred. Our definition of a response was a T_4 or T_3 concentration at least 10% higher than that of the three preceeding samples and a progressive increase in the concentrations of hormones in the three following samples.

The latency periods recorded in the present study were not corrected for the relatively short periods of time for transport through the afferent and efferent catheters and vessels. This is approximately 1 minute as judged from a number of experiments with infusion of Evans blue.

The TSH employed was bovine TSH – the international standard preparation, a gift from the Medial Research Council,

0018-5043/78 0232-0152 $05.00

London. cAMP, AMP, ATP and theophylline were purchased from Sigma Chemical Corp. St. Louis, U.S.A.

Results

The effect of 5 mM cAMP and 100 μU TSH per ml was compared in three perfusion experiments (Table 1, experiments 1-3). The latency periods and shape of the hormone release curves were very similar after stimulation with TSH and cAMP in all three experiments. Figure 1 shows the T_3 release curves in one of the experiments.

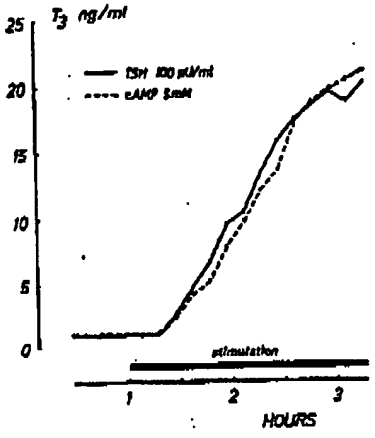

Fig. 1. The T_3 concentration in the effluent in a two-sided perfusion experiment. After one hour of perfusion with control medium one thyroid received a constant infusion of cAMP 5 mM while the other received TSH, 100 μU per ml.

In order to examine the dose-response relationship during cAMP stimulation, the effect of various concentrations of cAMP was examined. 5 mM and 1 mM cAMP were compared in three experiments (Table 1, experiments 4-6). The same pattern was observed in all three experiments. There was no difference in latency periods and the initial increase in hormone release was very similar after 1 and 5 mM cAMP. However, during the last part of the cAMP infusion the hormone release levelled off somewhat earlier on the side receiving 1 mM cAMP.

It was difficult to obtain a stimulating effect of cAMP when concentrations less than 1 mM were employed (Table 1). Lack of stimulation was seen after 0.2 mM (experiments 9 and 10), 0.5 mM (experiment 8) and 0.8 mM (experiment 11). In experiment 7 where 1 mM and 0.5 mM cAMP were compared a slight stimulating effect of 0.5 mM cAMP was observed. 1 mM induced an 8 fold increase while 0.5 mM induced a 2.5 fold increase in the hormone concentration in the effluent. Again there was no difference in the latency periods.

In all experiments where a stimulation of hormone release was observed the increase in T_4 and T_3 release started simultaneously. The shape of the release curves for T_4 and T_3 was rather similar except for a somewhat slower rise in the T_4 release during the initial part of the secretion phase. This is most easily visualized by recording the ratio between T_4 and T_3 in samples. Figure 2 shows the mean variation in this T_4/T_3 ratio in the experiments where 5 mM cAMP was infused. As can be seen, cAMP caused a temporary fall in the ratio reflecting a more rapid increase in the T_3 secretion during the initial phase of stimulation. 1 mM cAMP and 100 μU TSH per ml caused a similar variation in the T_4/T_3 ratio in effluent. Since the T_4/T_3 ratios in thyroid effluent during control perfusion and during prolonged stimulation were nearly identical only the T_4 values are given in Table 1.

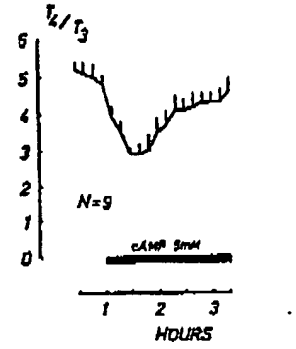

Fig. 2. The calculated T_4/T_3 ratio (weight/weight) in the effluent in nine perfusion experiments. After one hour of perfusion with control medium the thyroids received a constant infusion of cAMP, 5 mM, mean + SE.

The effect of the phosphodiesterase inhibitor theophylline was examined in three experiments (Table 1, experiments 12-14). Since theophylline interfered in the T_3 assay, only T_4 was measured in these experiments. 5 mM theophylline induced a moderate increase in the hormone release. The latency period and shape of the hormone release curve was comparable to that seen after cAMP. Figure 3 shows the T_4 release in one of the experiments.

In addition the effect of two other nucleotides ATP and AMP was investigated. ATP 1 or 5 mM, and AMP 5 mM had no stimulating effect (Table 1, experiments 14 and 15).

Discussion

In the present series of experiments exogenous cAMP and TSH in high concentrations induced the same pattern of hormone release from the thyroid. There was a simultaneous increase in the T_4 and T_3 release and during the early phase the rise in T_3 release was somewhat steeper than that of T_4.

When TSH, 100 μU per ml, and cAMP, 5 mM, were compared in two-sided perfusion experiments no differences in the duration of the latency periods were observed. Since high concentrations of TSH have been shown to increase the generation of cAMP in the

Table 1.

Experiment no.	Stimulant	Latency period* min	T_4 concentration in effluent** ng/ml	
			early	late
1	cAMP 5 mM	15	4.70	101.3
	TSH 100 μU/ml	15	3.97	116.3
2	cAMP 5 mM	20	1.40	24.9
	TSH 100 μU/ml	20	2.15	65.6
3	cAMP 5 mM	20	12.5	62.8
	TSH 100 μU/ml	20	10.2	135.7
4	cAMP 5 mM	20	3.57	70.2
	cAMP 1 mM	25	2.02	29.0
5	cAMP 5 mM	15	5.49	82.2
	cAMP 1 mM	15	3.87	39.9
6	cAMP 5 mM	20	5.58	61.7
	cAMP 1 mM	20	3.76	34.9
7	cAMP 1 mM	25	2.53	19.9
	cAMP 0.5 mM	25	2.02	4.90
8	cAMP 5 mM	20	1.76	13.8
	cAMP 0.5 mM	–	5.77	5.95
9	cAMP 5 mM	20	8.44	39.9
	cAMP 0.2 mM	–	6.70	6.43
10	cAMP 5 mM	15	2.12	47.6
	cAMP 0.2 mM	–	1.99	1.67
11	cAMP 0.8 mM	–	4.73	2.73
	control	–	5.70	3.70
12	theophylline 5 mM	25	1.88	3.60
	control	–	2.43	1.79
13	theophylline 5 mM	10	1.02	3.28
	control	–	0.98	0.69
14	theophylline 1 mM	–	2.76	2.78
	ATP 1 mM	–	3.37	2.04
15	ATP 5 mM	–	3.28	2.52
	AMP 5 mM	–	1.62	0.50

*Latency period: the time interval until (but *not* including) the first sample of the response which showed a definite increase.
**Mean of the three first and three last samples from each experiment.

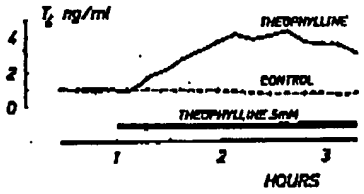

Fig. 3. The T_4 concentration in the effluent in a two-sided perfusion experiment. One thyroid acted as control while the other received a constant infusion of theophylline, 5 mM, starting after one hour of perfusion with control medium.

thyroid quickly (*Gilman and Rall* 1968, *Williams* 1972, *Kendall-Taylor* 1972, *Van Sande and Dumont* 1973, *Swillens, Van Sande, Pochet, Delbeke, Piccart, Paiva and Dumont* 1976) this finding is in accordance with the role of cAMP as an intermediate in the action of TSH on thyroid secretion.

In contradistinction to what we have found with TSH infusion (*Laurberg* 1977) we did not detect an increase in latency with infusions of decreasing concentrations of cAMP. Either no response was obtained or when an increase in the hormone release was induced, the latency period was of the same length as that observed after a strong stimulus.

Infusion of the phosphodiesterase inhibitor theophylline in a concentration of 5 mM induced an increase in the release of T_4. The stimulation, wich was presumably caused by accumulation of endogenous cAMP, was only moderate. However, the latency period was in the same order of magnitude as that following stimulation with high concentrations of TSH.

Provided cAMP is the mediator of TSH induced thyroid hormone secretion at all concentrations of TSH, the findings presented here seem to indicate, that the

fluent**

late

101.3
116.3
24.9
65.6
62.8
135.7
70.2
29.0
82.2
39.9
61.7
34.9
19.9
4.90
13.8
5.95
39.9
6.43
47.6
1.67
2.73
3.70
3.60
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3.28
0.69
2.78
2.04
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0.50

owed a definite increase.

have found with TSH not detect an in- of decreasing concen- onse was obtained one release was in- the same length as ulus.

e inhibitor theophyl- induced an increase tion, wich was pre- of endogenous cAMP, e latency period was as that following sti- ns of TSH.

of TSH induced thy- ncentrations of TSH, to indicate, that the

processes responsible for the time-lapse between addition of TSH and augmentation of thyroid hormone release can be divided into two groups. Early processes contribute insignificantly to the latency period when high concentrations of TSH are employed, but are responsible for the prolongation of the latency period observed when the thyroid is stimulated by low concentrations of TSH. In the process sequence leading to thyroid hormone secretion this 'variable' latency period is confined to the interval starting with TSH-receptor interaction and terminating with generation of cAMP. The later time-consuming processes, i.e. after cAMP generation, seem to have a fixed duration. In a recent study of *Ketelbant-Ballasse, Van Sande, Neve and Dumont* (1976) employing scanning electron microscope examination of open follicles in dog thyroid slices, it was possible to demonstrate generation of lamellopodia and pseudopods 2 minutes after addition of high concentrations of TSH. Accordingly the 'obligatory' latency period may be related mainly to processes such as generation of colloid droplets, hydrolysis of thyroglobulin and transport of free thyronines to the vascular space. The results of the present study suggest that the duration of these processes is independent of the degree of stimulation.

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References

Dumont, J.E.: The action of thyrotropin on thyroid metabolism. Vitam.Horm. 29: 287-412 (1971)

Field, J.B.: Thyroid-stimulating hormone and cyclic adenosine 3',5'-monophosphate in the regulation of thyroid gland function. Metabolism 24: 381-393 (1975)

Gilman, A.G., T.W. Rall: Factors influencing adenosine 3',5'-phosphate accumulation in bovine thyroid slices. J.biol.Chem. 243: 5867-5871 (1968)

Kendall-Taylor, P.: Comparison of the effects of various agents on thyroidal adenyl cyclase activity with their effects on thyroid hormone release. J.Endocr. 54: 137-145 (1972)

Ketelbant-Ballasse, P., J. Van Sande, P. Neve, J.E. Dumont: Time sequence of 3',5'-cyclic-AMP accumulation and ultrastructural changes in dog thyroid slices after acute stimulation by TSH. Horm.Metab.Res. 8: 212-215 (1976)

Laurberg, P.: T_4 and T_3 release from the perfused canine thyroid isolated in situ. Acta endocr. (Kbh.) 83: 105-113 (1976)

Laurberg, P.: The relative contribution of thyroxine and triiodothyronine to the hormone secretion from the perfused canine thyroid during various degrees of stimulation. Endocrinology 100: 656-662 (1977)

Swillens, S., J. Van Sande, R. Pochet, D. Delbeke, M. Piccart, M. Patva, J.E. Dumont: Kinetics of adenosine 3',5'-monophosphate accumulation in dog thyroid slices. Eur.J.Biochem. 62: 87-93 (1976)

Van Sande, J., J.E. Dumont: Effects of thyrotropin, prostaglandin E_1 and iodide on cyclic 3'5'-AMP concentration in dog thyroid slices. Biochim.Biophys.Acta 313: 320-328 (1973)

Weeke, J., H. Ørskov: Ultrasensitive radioimmunoassay for direct determination of free triiodothyronine concentration in serum. Scand.J.clin.Lab.Invest. 35: 237-244 (1975)

Weeke, J., H. Ørskov: Evaluation of thyroid function. In: K.G.M.M. Alberti (ed.). Recent Advances in Clinical Biochemistry, Vol. 1. Churchill, Livingstone, London, pp. 111-128 (1978)

Williams, J.A.: Cyclic AMP formation and thyroid secretion by incubated mouse thyroid lobes. Endocrinology 91: 1411-1417 (1972)

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